

Comparative expression analysis of two sugarcane polyubiquitin promoters and flanking sequences in transgenic plants

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Summary

GUS (*uidA*) reporter gene expression for two sugarcane polyubiquitin promoters, *ubi4* and *ubi9*, was compared to expression from the maize *Ubi-1* promoter in stable transgenic rice (only *ubi9*) and sugarcane (*ubi4* and *ubi9*). *Ubi9* drove high-level GUS expression, comparable to the maize *Ubi-1* promoter, in both callus and regenerated plants of rice transformed by *Agrobacterium*. This high level expression was inherited in R1 plants. Expression from *ubi4* and *ubi9* was quite high in sugarcane callus transformed via particle bombardment. Expression dropped to very low or undetectable levels in the resulting plants; this drop in expression resulted from PTGS. PTGS in regenerated sugarcane plants also occurred with the maize *Ubi-1* promoter. In sugarcane callus, *ubi4* was HS inducible, but *ubi9* was not. This physiological difference corresponds to a MITE insertion that is present in the putative HSEs of *ubi9* but not present in *ubi4*.

Key words: gene expression – monocot – promoter – PTGS – rice – sugarcane

Abbreviations: GUS = β -glucuronidase. – HS = heat shock. – HSE = heat shock element. – MARs = nuclear matrix attachment regions. – MITE = miniature inverted-repeat transposable element. – NOS = nopaline synthase terminator. – *npII* = neomycinphosphotransferase II. – PTGS = post-transcriptional gene silencing. – UTR = untranslated region. – WT = wild-type

Introduction

We previously reported the isolation of two polyubiquitin genes, *ubi4* and *ubi9*, from sugarcane and characterization of these promoters in transient expression assays in tobacco,

sugarcane (Wei et al. 1999), maize, sorghum, banana, pineapple, and garlic (Wei 2001). Our purpose was to increase the number of constitutive promoters available for monocot transformation. By constitutive we mean high levels of expression in most or all plant organs in the absence of an inducing stimulus, not necessarily equal expression in every organ. We now report further characterization of these promoters in stable transgenic sugarcane and rice.

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The maize polyubiquitin promoter *Ubi-1* (Christensen et al. 1992) has been tested with transient assays in a wide range of monocot species (Wilmink et al. 1995, Christensen and Quail 1996), including rice (Cornejo et al. 1993) and sugarcane (Gallo-Meagher and Irvine 1993). In both rice and sugarcane, these assays found that *Ubi-1* produced more expression foci than other tested promoters, including the rice actin Act1 promoter (McElroy et al. 1990, McElroy et al. 1991), the pEmu promoter (Last et al. 1991, Chamberlain et al. 1994), and some configurations of the Cauliflower Mosaic Virus 35S promoter. Additionally *Ubi-1* has been used to produce stable transgenic rice (Cornejo et al. 1993) and sugarcane (Gallo-Meagher and Irvine 1996, Ma et al. 2000). For these reasons, *Ubi-1* is often used as a standard of comparison when characterizing new monocot promoters (Schenk et al. 1999, Wang et al. 2000, Schenk et al. 2001). We also chose the maize *Ubi-1* promoter as a standard of comparison.

In rice the sugarcane ubi9 promoter and the maize *Ubi-1* promoter produced numerous callus lines that expressed β -glucuronidase (GUS) at high levels. These high levels were maintained (or increased) in regenerated plants and through the R1 generation. In sugarcane both of the sugarcane ubi promoters and the maize *Ubi-1* promoter produced numerous callus lines that expressed GUS at high levels. In regenerated sugarcane plants, however, all of the GUS reporter constructs were silenced. To determine what type of silencing had occurred, nuclear run-off experiments were conducted to assay transcription of GUS and several other genetic elements.

The maize *Ubi-1* promoter is induced by heat shock (HS); however, the sugarcane gene sub-family containing the ubi4 and ubi9 genes appears not to respond to HS (Wei et al. 1999). Transgenic sugarcane callus lines containing all three of these promoters were tested for induction of GUS expression by HS.

Analysis of the nucleotide sequence flanking the ubi4 gene revealed the presence of numerous motifs associated with nuclear matrix attachment regions (MARs). To test whether these elements had any effect on transgene expression, GUS expression constructs with or without upstream and downstream putative MARs were compared in transgenic rice lines.

Materials and Methods

Plasmid construction

Expression constructs pubi4-GUS and pubi9-GUS contain the indicated «promoter» (including its 5' untranslated region [UTR] and intron) driving a GUS reporter gene (Jefferson et al. 1986) followed by a nopaline synthase (NOS) terminator in pUC19, as previously described (Wei et al. 1999). Intron deletion cassettes ubi4 Δ I-GUS and ubi9 Δ I-GUS were made by replacing the *Hind*III-*Xba*I fragments containing the promoter, 5' UTR, and intron of ubi4-GUS and ubi9-GUS, respectively, with PCR fragments containing the same promoter and 5' UTR, followed immediately by an *Xba*I site. Intron deletion con-

structs were not used for plant expression experiments reported here, but fragments of them were used as probes in the nuclear run-off experiments (see below). pAHC27 (*Ubi-1*-GUS) is a similar GUS expression construct that uses the maize *Ubi-1* promoter (also including its 5' UTR and intron) (Christensen and Quail 1996).

Binary plasmids pCAM-ubi9-GUS and pCAM-*Ubi-1*-GUS were made by inserting *Hind*III-*Eco*RI fragments containing the ubi9-GUS and *Ubi-1*-GUS expression cassettes into pCambia1300 (www.cambia.org).

pCAM-5'M-ubi9-GUS-3'M adds putative MARs from the ubi4 gene to the ubi9-GUS cassette in pCAM-ubi9-GUS (see Fig. 1 for expression cassettes). A 2 kb fragment beginning just downstream of the stop codon and including the putative 3' MAR was amplified (upstream primer: GCACGTCGACAGCTGTCCTCCAGGTTCAC, downstream primer: ATTGAATCCGGCGCTACACTGGCTGTTC). The PCR product was blunt-end cloned into the *Srf*I site of pCRScript (Stratagene) and orientation confirmed by PCR. This fragment was excised as a *Sac*I-*Eco*RI fragment and used to replace the *Sac*I-*Eco*RI fragment containing the NOS terminator of pCAM-ubi9-GUS, forming pCAM-ubi9-GUS-3'M. A 2.3 kb fragment containing a putative MAR upstream of the ubi4 gene was inserted into the *Hind*III and *Eco*RI sites of pBluescript II KS+ (Stratagene). This fragment was then released with *Kpn*I and *Xba*I and cloned into the same sites of pGEM-4Z (Promega). The insert was then released as a *Hind*III-*Hind*III fragment and cloned into the *Hind*III site 5' of the ubi9 promoter in pCAM-ubi9-GUS-3'M, forming pCAM-5'M-ubi9-GUS-3'M. Orientation of the 5' MAR containing insert was confirmed by PCR.

PCR for expression constructs was performed with the Expand High Fidelity PCR (Boehringer) mix of polymerases per manufacturer's instructions to reduce the likelihood of errors in amplification.

Plant transformation and growth

Rice

Agrobacterium (EHA 105) -mediated transformation of rice (*Oryza sativa* cv. Taipei 309) was performed with mature seed, scutellum-derived callus as described (Hiei et al. 1994). Selection was performed on 2N6 media (Hiei et al. 1994) containing 50 μ g/mL hygromycin B (Sigma) and 250 μ g/mL cefotaxime (Agri-Bio) to eliminate *Agrobacterium*. After regeneration, plants were transferred to pots immersed in tubs of water in a greenhouse.

Sugarcane

Embryogenic sugarcane calli (*Saccharum* hybrid cv. H62-4671) were bombarded as described (Ma et al. 2000). GUS reporter constructs were co-bombarded with pHA9, a selection plasmid that contains the neomycinphosphotransferase II (*nptII*) gene driven by the maize *Ubi-1* promoter. Bombarded calli were initially placed on MS1 (Ma et al. 2000) plates without selection. After 5–7 days, calli were transferred to MS1 plates with 50 μ g/mL G418 (Agri-Bio). After four weeks, calli were transferred to MS1 plates with 100 μ g/mL G418. G418 resistant calli were proliferated on MS1 media and then placed on MS0 (Ma et al. 2000) media for regeneration. Regenerated plants were transferred to pots in a greenhouse and then to a field test plot.

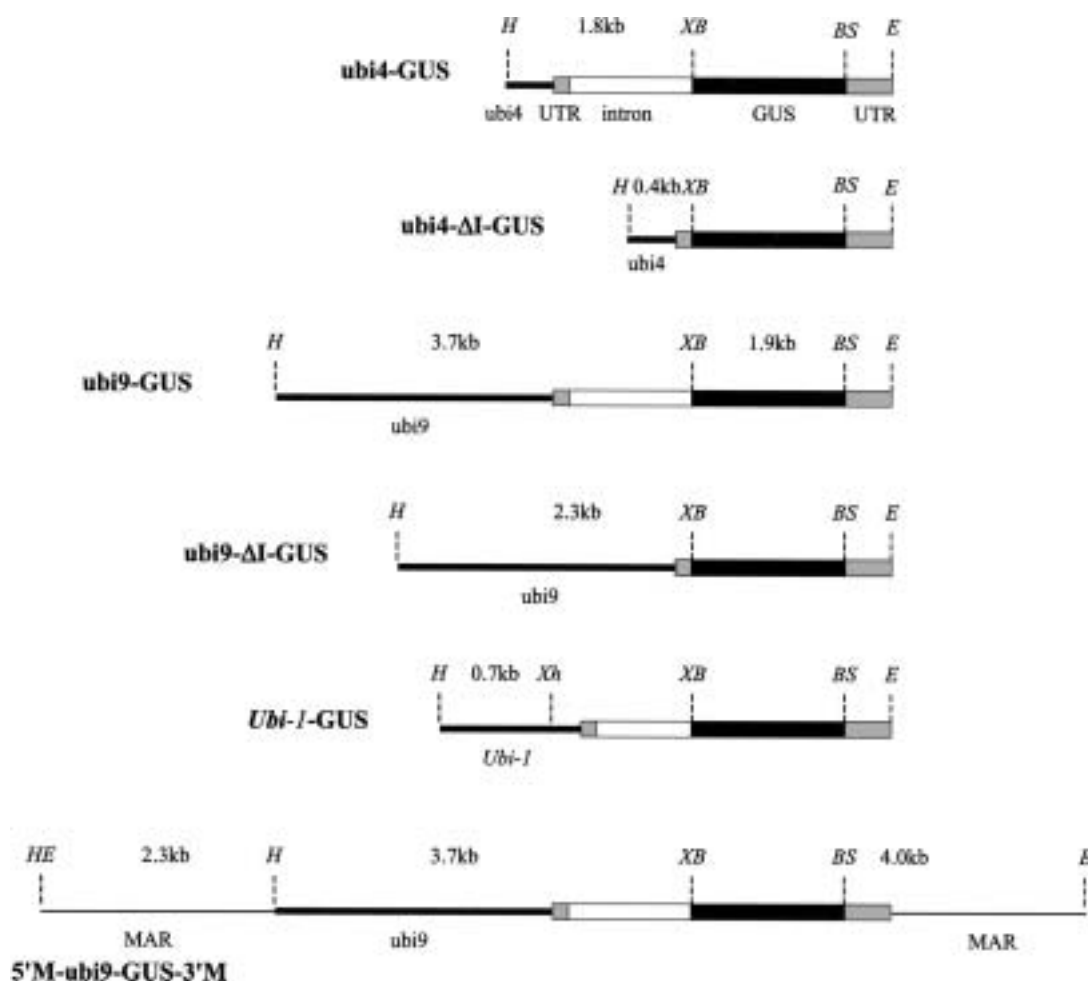


Figure 1. GUS expression cassettes. Some cassettes were used in more than one vector, e.g. *pubi9-GUS* contains the *ubi4-GUS* expression cassette in a pUC plasmid, while *pCAM-ubi9-GUS* contains the same cassette in a CAMBIA binary plasmid. *B*, *Bam*HI; *H*, *Hind*III; *S*, *Sac*I; *X*, *Xba*I; *Xh*, *Xho*I.

Histochemical GUS staining

Plant tissue was incubated in GUS staining solution containing 50 mmol/L sodium phosphate buffer, pH 7.4, 10 mmol/L EDTA, 0.1 % Triton X-100, and 2 mmol/L X-Gluc (Clontech, #8080-2) for 12–16 h at 37 °C. Leaf tissue in staining buffer was briefly placed under vacuum several times to aid substrate penetration. Chlorophyll was extracted from green tissues in 70 % ethanol after incubation in GUS staining solution.

GUS activity assays

Quantitative analysis of GUS activity was performed using the GUS-Light kit (Tropix) according to the manufacturer's protocol. Purified *E. coli* GUS (Sigma, cat. # G7396) was used as a standard. Chemiluminescence was quantified on an MLX microplate luminometer (DYNEX). Protein quantification (Bradford 1976) was performed using the Bio-Rad Protein Assay Kit II (500-0002) with BSA as a standard. Average values, from multiple samples and replicate measurements,

and standard deviations were calculated with Microsoft Excel. Data for expression after HS were presented as percent change. Absolute expression values before and after HS were analysed by Student's *t* test to determine if the observed changes were significant at $P < 0.05$.

Quantitation of NPTII protein

Crude protein extracts were prepared by grinding approximately 100 mg of leaf tissue in liquid nitrogen and mixing with 0.5 mL of extraction buffer containing 10 mmol/L sodium sulfite, 2 % (w/v) polyvinylpyrrolidone (average mol. wt. 40,000), 3 mmol/L sodium azide, and 2 % Tween-20 in 1X PBS-T (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L NaHPO₄, and 2 mmol/L KH₂PO₄). The samples were centrifuged at 20,000 g for 15 min at 4 °C. Concentration of NPTII protein in the supernatant was determined by ELISA using the PathoScreen kit for NPTII (Agdia psp7300) according to manufacturer's protocol. Protein quantification (Bradford 1976) was performed using the Bio-Rad Protein Assay Kit II (500-0002) with BSA as a standard.

Nuclear run-off assays

Nuclei were isolated from the leaves of field-grown sugarcane plants and run-offs performed as previously described (Ingelbrecht and de Carvalho 1992). Approximately 2×10^6 nuclei were used per run-off assay. Concentration of nuclei was estimated by purifying DNA from an aliquot of nuclei and determining the OD₂₆₀. Each sugarcane nucleus was assumed to contain 8 pg DNA (Bennett and Smith 1976). Slot blots were prepared by applying purified (GeneClean Spin, BIO101) DNA fragments (1 µg per slot) to Hybond-N⁺ membranes (Amersham) following the manufacturer's recommendations. Probe DNA was gel purified for the sequence of interest. The *ubi4* promoter probe was a 0.4 kb *HindIII*-*XbaI* fragment from *ubi4-ΔI*-GUS (Fig. 1); the *ubi9* promoter probe was a 2.3 kb *HindIII*-*XbaI* fragment from *ubi9-ΔI*-GUS (Fig. 1). The *ubi4* and *ubi9* probes did not include intron sequence but did include the 64 bp 5' UTR. The *Ubi-1* promoter probe was a 0.7 kb *HindIII*-*XhoI* fragment from pAHC27 (see *Ubi-1*-GUS, Fig. 1), which contains only upstream sequence with no 5' UTR or intron. The GUS probe was a 1.9 kb *Bam*HI-*Bam*HI fragment from *ubi9*-GUS (Fig. 1); the *nptII* probe was a 0.8 kb *Sall*-*Sall* fragment containing only the *nptII* gene from pHA9 (see above); the pUC probe was linearized pUC19; the *nos* 3' probe was a 0.26 kb PCR product amplified from *ubi4*-GUS. A 1.2 kb *EcoRI*-*EcoRI* insert from scubi 561, a sugarcane polyubiquitin cDNA (Albert et al. 1995), was used as an endogenous control. Ubiquitin coding sequence is highly conserved so the scubi561 probe is expected to hybridize to transcripts from all members of the polyubiquitin gene family. After hybridization and stringency washes, blots were exposed to Marsh Blue Sensitive autoradiograph film (Marsh Bioproducts) with 2 Lightning Plus intensifying screens (DuPont 200224) for 48 to 72 h at -80 °C.

Results

Expression in transgenic rice

GUS reporter constructs pCAM-*ubi9*-GUS and pCAM-*Ubi-1*-GUS were introduced into rice calli by co-cultivation with *Agrobacterium*. Nine hygromycin^R callus lines harboring *ubi9* (seven lines) and *Ubi-1* (two lines) were chosen for GUS expression analysis. With the exception of four lines (*ubi9* lines 6, 9, 16, and 30, which were not tested), these lines were judged to be independent transformation events, based on Southern analysis (data not shown). Quantitative analysis of GUS expression in these callus lines showed that the strongest line accumulated GUS protein to approximately 0.1 % of soluble protein, with several lines accumulating 0.05 % or greater (Fig. 2A).

Not all rice callus lines were successfully regenerated. GUS expression in leaves, stems, and roots was analyzed when regenerated plants grew to 3 months old (Fig. 2B). Comparison of the relative expression levels revealed similar ranking among calli and plant lines; however, GUS expression in differentiated tissues was more than ten-fold higher than that in calli when normalized to total soluble protein. On a protein basis, roots accumulated more GUS than leaves or stems; however, the majority of this difference results from the lower levels of total protein in roots.

Several lines were grown to maturity, and seeds were grown to assay GUS expression in the R1 generation. GUS expression in all these lines remained high, with one line (*ubi9* line #30) producing 2.6 %, 0.7 %, and 0.8 % of soluble protein in roots, stems, and leaves, respectively.

Expression in transgenic sugarcane

GUS reporter constructs *pubi4*-GUS, *pubi9*-GUS, and pAHC27 were introduced into embryogenic sugarcane callus by particle bombardment. The great majority of calli surviving selection showed GUS activity detectable by histochemical staining. Many of these produced uniform dark blue staining. Seventeen GUS⁺ callus lines for each *pubi4*-GUS and *pubi9*-GUS construct and nine GUS⁺ callus lines with pAHC27 were chosen for expression analysis. These lines are thought to be independent; however, because of the large number of unresolved bands on genomic Southern (data not shown), this was not confirmed for all lines. Quantitative expression of the GUS reporter gene in each transgenic callus line was determined soon after recovery (Fig. 3). The strongest line accumulated GUS protein to approximately 0.5 % of soluble protein, with numerous lines accumulating greater than 0.1 %.

Plants were successfully regenerated from the majority of the transgenic sugarcane lines. Histochemical staining was then employed to monitor expression of the GUS reporter gene during plant development. GUS activity was detectable in the young seedlings grown *in vitro* but progressively decreased to very low levels after the seedlings were planted in soil and transferred to the greenhouse. Subsequent examinations during 9 months of greenhouse growth and more than 6 months of field growth found that GUS activity remained at very low or undetectable levels, with the highest line accumulating GUS at 0.0003 % of total protein. Apparently some form of transgene silencing was triggered in all of these sugarcane lines during or after plant regeneration.

Accumulation of neomycin phosphotransferase II protein (NPTII) was determined in leaf tissues of 38 mature, field-grown sugarcane lines, including 21 lines produced for the present study. For each of these lines, maize *Ubi-1* was used to drive the *nptII* gene. NPTII accumulation was below 0.1 % of total protein for all of these lines, with only two lines averaging more than 0.05 %, and many lines below the limits of detection. Individual leaf samples from three lines did exceed 0.1 % (the highest being 0.3 %); however, these relatively high levels were not reproduced in other samples from the same line. Each sugarcane plant (or stool) consists of numerous stems (or culms). Variation between samples from the same plant may represent sectors of expression/silencing or may be a non-systemic stress response, as has been previously observed with the *Ubi-1* promoter (Takimoto et al. 1994). Overall, accumulation of NPTII was quite low in mature plants. Quantitative assays of NPTII accumulation were not done while these lines were in callus stage, so we do not know

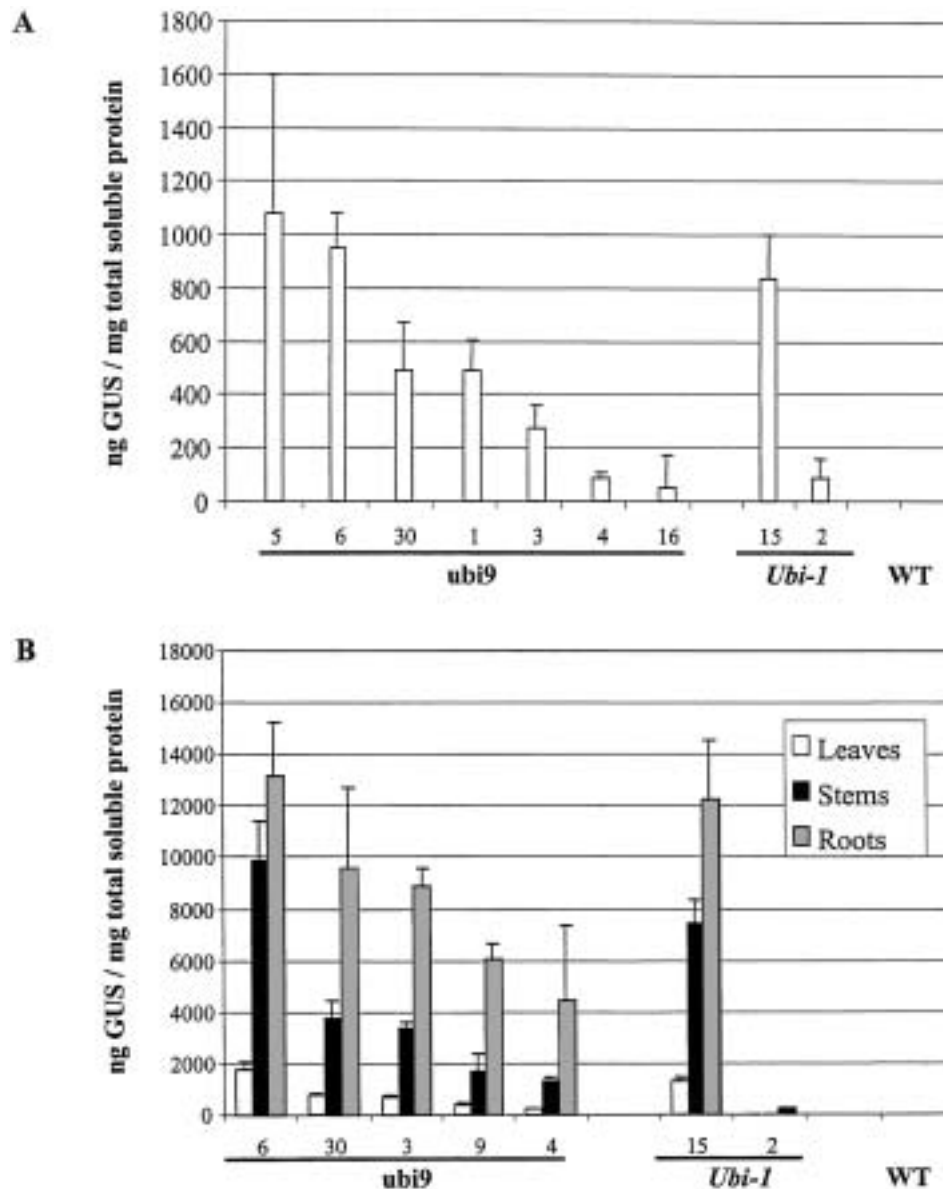


Figure 2. Expression of GUS in callus (A) or leaves and roots of rice plants (B). All values are the mean of 2–8 tissue samples, 2 replicate luminescence measurements for each sample. Error bars indicate standard deviation.

whether the low levels observed in plants represent a decrease from callus expression, as was observed for GUS.

Nuclear run-off assays in sugarcane

Nuclear run-off assays showed that the GUS and *nptII* transgenes were being transcribed in all five tested, silenced, transgenic lines (Fig. 4). As transgene transcription is occurring at a significant rate when compared to the endogenous polyubiquitin genes, the low level or absence of transgene protein indicates that the silenced plant lines have undergone post-transcriptional gene silencing (PTGS). In addition to the

GUS gene, pUC vector sequences and in some lines the *Ubi-1* promoter (present in the selection cassette of all lines) were also transcribed. Because the *ubi4* and *ubi9* promoter probes contained the 5' UTR sequence, we cannot determine whether the hybridization to these probes may represent transcription of promoter sequences. This is consistent with complex and/or rearranged transgene loci, wherein sequences not intended to be transcribed become inserted downstream of transgene or endogenous promoters. Transcription of the *nos* 3' region, present on both the GUS and *nptII* genes, appeared to be very low or undetectable. This may be due to the short length of this fragment or unknown factors causing poor hybridization. Transcription of vector and promoter se-

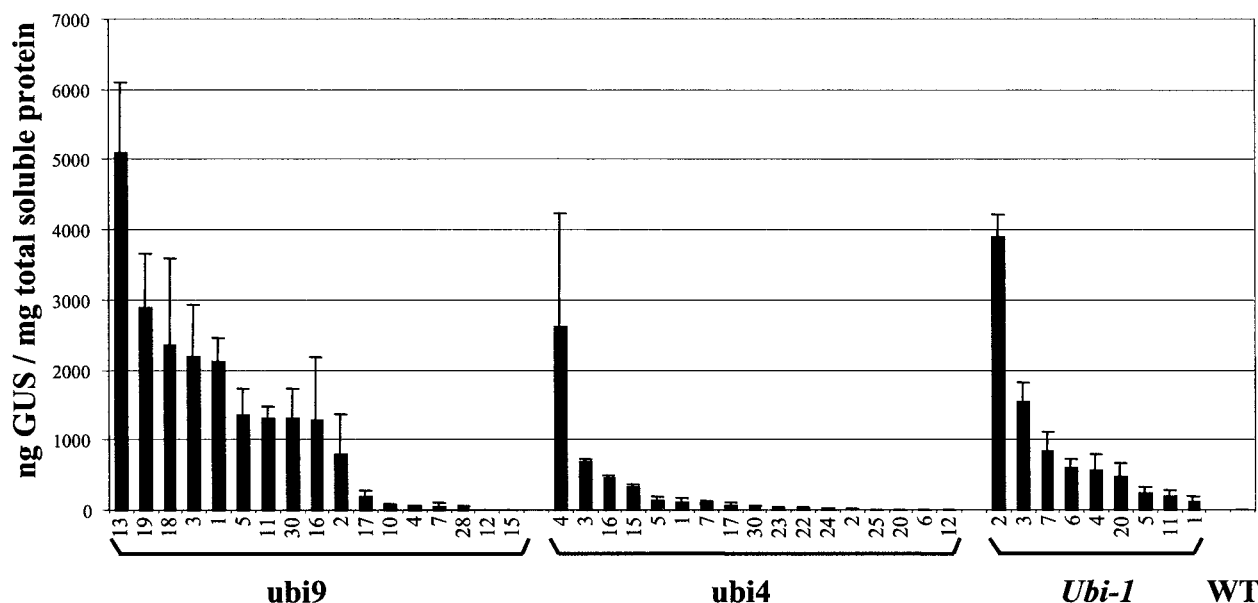


Figure 3. Expression of GUS in sugarcane callus. All values are the mean of 2–8 tissue samples, 2 replicate luminescence measurements for each sample. Error bars indicate standard deviation.

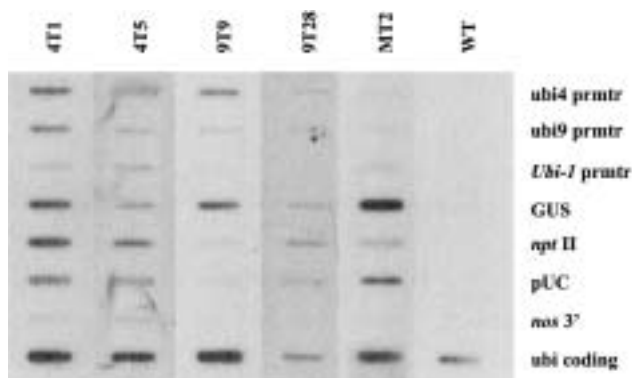


Figure 4. Nuclear run-offs from transgenic and non-transgenic (WT) H62-4671 sugarcane lines.

quences is consistent with «read-through» transcription, which could result in self-complementary transcripts from tail-to-tail transgene insertions. The resulting double-stranded RNA would be likely to trigger PTGS (Chuang and Meyerowitz 2000).

Effect of HS on transgene expression

Transgenic sugarcane calli growing at 26 °C were moved to 37 °C or 42 °C for 2 h, and then assayed for GUS activity. Fig. 5 A shows the relative change of GUS activity following HS treatment. In sugarcane calli, GUS expression driven by the *ubi4* promoter increased substantially, with one line increased 650 % and an average of 185 %, after HS. In three of the five tested *ubi4* lines and in three of the four tested *Ubi-1*

lines, the increase in expression after HS was significant (Student's *t* test, $P < 0.05$). In contrast the *ubi9* promoter did not produce increased GUS expression after HS, but rather showed a small decrease; two lines showed no significant change and two lines showed a significant decrease. The HS induction of GUS expression in *ubi4* lines was similar to the induction seen in lines containing *Ubi-1*, which has been shown to be HS inducible (Christensen et al. 1989, 1992).

To test if this HS response occurs in rice plants, four *ubi9* lines were moved from 26 °C to 42 °C for 2 h. Average GUS expression was substantially reduced after HS--- in some cases the decrease was significant, in others it was not; however, none of the lines showed an increase in GUS expression after HS (Fig. 5 B). This supports the hypothesis that the *ubi9* promoter is not HS inducible.

Previously, we determined that the mRNA pool from the polyubiquitin gene sub-family that includes *ubi4* and *ubi9* is not significantly altered by HS (Wei et al. 1999). At first glance this appears to contradict the HS expression results of the present experiments. However, if some members like *ubi4* of the sub-family are up-regulated while others like *ubi9* are down-regulated, the result could be little net change in the combined mRNA pool.

The maize *Ubi-1* promoter is HS inducible and is described as having two overlapping HS element (HSE) consensus sequences (Christensen et al. 1992). This determination of the HSE consensus sequence (Pelham 1982) is 15 bp long. Later work characterized functional HSEs as consisting of 5 bp modules in alternating palindromic orientation, with a minimum of three modules required for activity, and an «invariant» G in position 2 of the module. In some cases spacing between modules was found critical for function (Scharf et al.

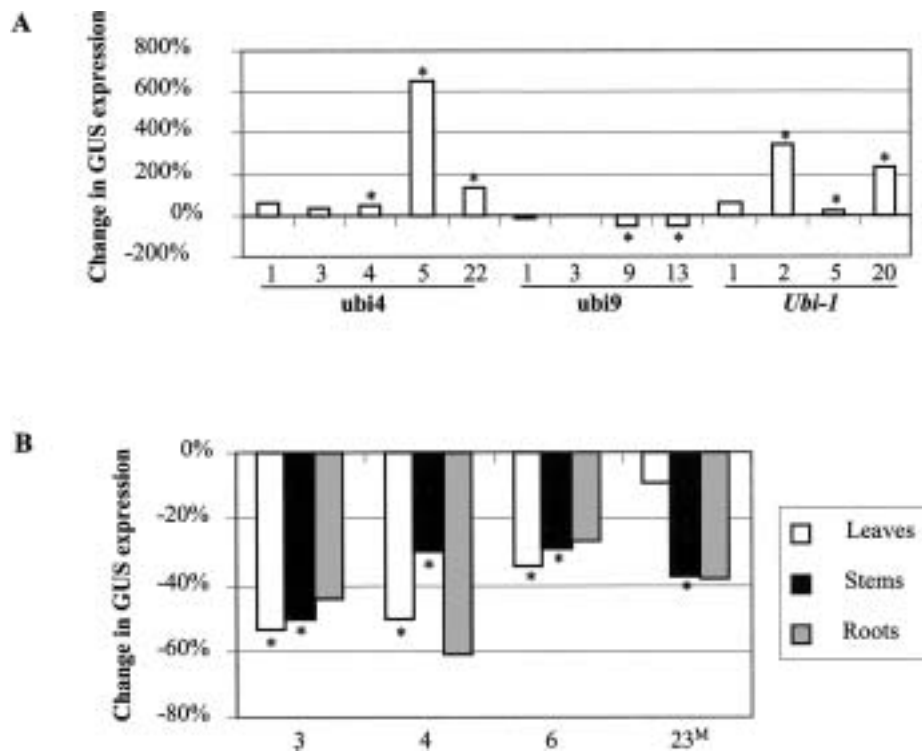


Figure 5. Percent change in GUS expression after HS treatment in sugarcane callus lines (A) containing GUS controlled by the indicated promoter, and rice plant lines (B). * indicates significant change at $P < 0.05$. All rice plant lines contain ubi9-GUS. ^M contains pCAM-5'M-ubi9-GUS-3'M, see Methods.

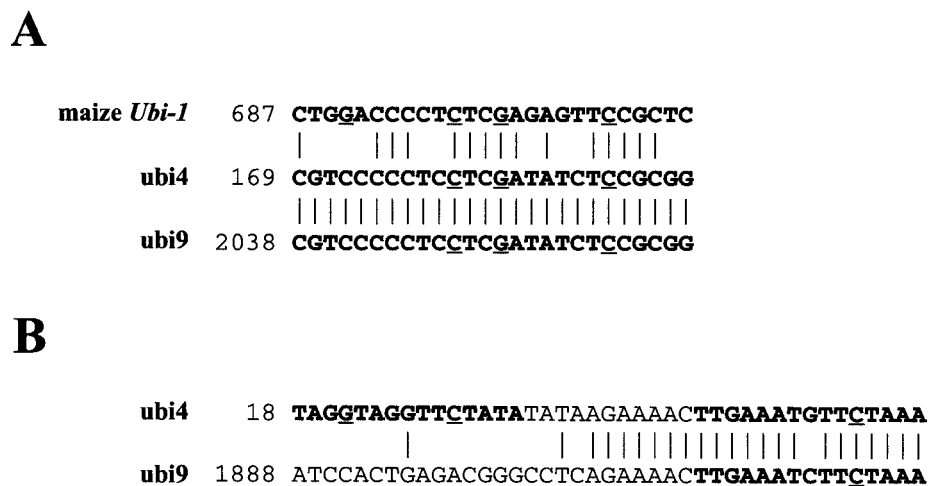


Figure 6. A. Alignments of putative overlapping HSEs in *Ubi-1*, *ubi4* and *ubi9*. B. Alignments of putative non-overlapping HSE's in *ubi4* and *ubi9*. Bold letters indicate the putative HSEs, invariant G/Cs are underlined.

1994). By aligning *ubi4* with *Ubi-1*, a good match to the overlapping HSEs can be found (Fig. 6). In the *Ubi-1* HSEs, all four of the «invariant» G/C residues are present; three are present in *ubi4* and *ubi9*. Because this region is 100 % identical in *ubi4* and *ubi9*, it seems inconsistent with the different HS response from the two promoters in transgenic calli. Further comparison of the two sugarcane promoters revealed two ad-

ditional possible HSEs further upstream in *ubi4*. These potential HSEs show higher identity (67 % and 80 % vs. 47 % and 47 %) to the HSE consensus sequence as defined by (Pelham 1982). Again three invariant G/C residues are present. Unlike *Ubi-1*, the two HSEs are not overlapping, with 11 intervening nucleotides. Comparison of this region of *ubi4* to a corresponding region of *ubi9* finds 92 % identity for the down-

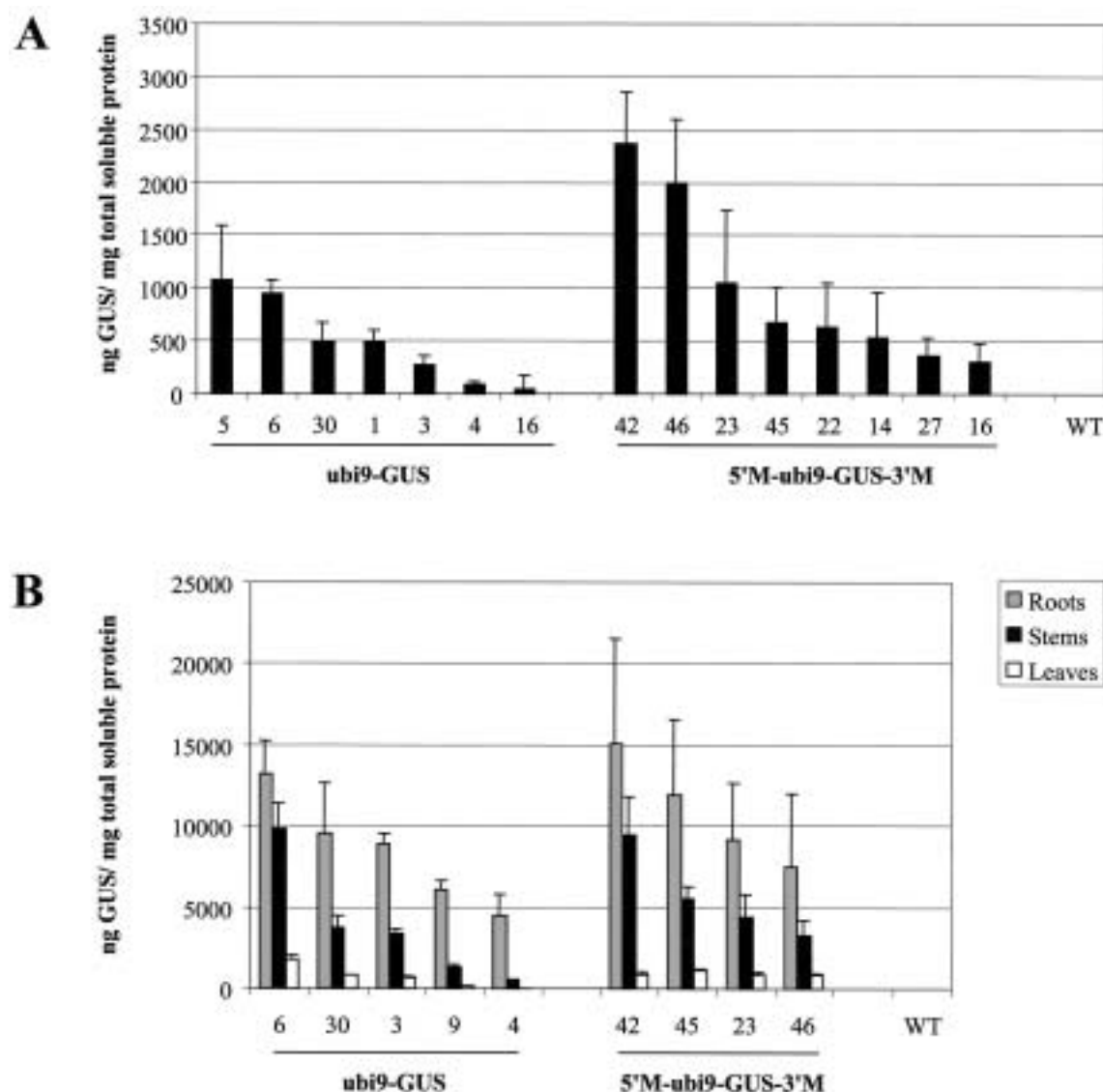


Figure 7. Effect of MARs on expression of GUS in rice. A. Callus lines. B. Plant lines. All values are the mean of 2–8 tissue samples, 2 replicate luminescence measurements for each sample. Error bars indicate standard deviation.

stream HSE and the downstream part of the 11 intervening bases. Upstream from this point, however, the nucleotide identity ceases abruptly. This point marks the downstream end of an approximately 200 bp miniature inverted-repeat transposable element (MITE) previously described in the *ubi9* promoter (Wei et al. 1999). Immediately upstream from this MITE is another possible HSE, 80 % identical to the upstream *ubi4* HSE. However, this HSE contains only one of the invariant G/C residues. The combination of this point mutation and the significantly altered spacing between the HSEs resulting from the MITE insertion may explain the loss of HS induction in the *ubi9* promoter.

Effect of putative MARs on transgene expression

Analysis of the *ubi4* genomic sequence with «MAR-Finder» (Singh et al. 1997, <http://www.futuresoft.org/MAR-Wiz/>) revealed regions both upstream and downstream of the gene containing numerous motifs associated with MARs. An expression construct, pCAM-5'M-*ubi9*-GUS-3'M (see Methods and Fig. 1), flanking the *ubi9*-GUS cassette with these putative MARs was assembled and used to transform rice.

Quantitative assays of GUS expression were performed on callus (Fig. 7A) and R0 plants from the four lines that were regenerated (Fig. 7B) and compared to rice lines expressing

the ubi9-GUS construct without MARs. The average concentration of GUS protein in callus lines containing MARs was 102 % greater than in lines lacking MARs. However, because of the wide overlapping range in levels of expression, the difference was not significant at $P = 0.05$. The average GUS concentrations in the MARs-containing regenerated plant lines were also higher than in lines without MARs, but the difference was smaller than that seen in callus. In callus, variation between independent lines was not significantly affected by use of the putative MARs, with the coefficient of variation (cv) for lines without MARs at 0.82 and for those with MARs at 0.78. In differentiated plants, cv. values for lines with and without MARs were 0.99 and 0.15 in leaves, 0.97 and 0.47 in stems, and 0.40 and 0.30 in roots, respectively. Even with this small population size, the difference in cv. values for leaves was significant at $P = 0.01$. For callus, stems, and roots the differences were not significant, even at $P = 0.05$. It is possible that the MAR-like sequences flanking the ubi4 gene may provide some increase in expression and/or reduced variation between independent lines; however, our data do not clearly support this.

Discussion

In summary, these sugarcane polyubiquitin promoters were found to produce high levels of transgene expression in rice and sugarcane callus lines. In rice, where transformation was performed by *Agrobacterium*, this was also true in regenerated plants, and this high level of expression was inherited and remained stable through the R1 generation. In sugarcane plants, transgenes driven by these promoters underwent PTGS; however, this was also observed with the maize *Ubi-1* promoter.

Hansom et al. (1999) found that transgenic sugarcane lines using the maize *Ubi-1* promoter undergo PTGS less frequently than lines using other promoters; however, the measure of silencing they used is different from that used in our analysis. Using firefly luciferase as a reporter gene, they reported a range of luciferase expression from approximately 1,500 fg/ μ g protein to 50,000 fg/ μ g protein in plant lines scored as not silenced. This range means these lines accumulate luciferase at 0.00015 % to 0.005 % of total protein. This range is for the highest expressing organs in each line, with some organs in some lines at near zero.

While some of our sugarcane lines accumulated no detectable GUS protein after plant regeneration, others accumulated GUS up to 0.0003 % of total protein. Because this level was such a significant decrease from the levels observed in callus, we considered all the sugarcane plant lines to be silenced. However, the range for accumulation of GUS in our silenced lines overlapped with the range of accumulation of luciferase in the sugarcane plant lines scored as «not silenced» by Hansom et al. (1999). In our small sample, *Ubi-1*-GUS (pAHC27) lines frequently underwent silencing, in the

sense of a significant decrease in GUS expression from callus levels («developmental»), and also in the sense that these plant lines accumulated very low levels of GUS even though the GUS gene was transcribed at a significant rate. Because we did not assay NPTII accumulation in sugarcane callus, we cannot say that we have observed silencing for *Ubi-1-nptII* genes in the «developmental» sense. However, we did find low levels of expression in mature plants: 36 of 38 tested lines accumulated NPTII protein to less than 0.05 % of TSP. Numerous lines were below our detection threshold for NPTII protein. As these lines were originally selected as callus on G418 antibiotic, we think it likely that at least some of these lines did undergo a reduction in NPTII accumulation after plant regeneration. And for four of five tested lines (4T1, 4T5, 9T28, MT2, Fig. 4) transcription occurred at a significant rate when compared to the endogenous control, suggesting that low levels of NPTII accumulation are due to PTGS. While accumulation of both GUS and NPTII protein was low in mature sugarcane plants, the highest level of NPTII was approximately 100-fold higher than the highest level of GUS. It is possible that this reflects a difference between the promoters, but as there was no significant difference in GUS expression between the promoters, we feel it more likely the GUS vs. NPTII difference reflects some difference in the genes themselves (e.g., susceptibility to PTGS) or relative stability of the proteins.

We are aware that sugarcane lines that produce high levels of foreign protein (0.5–1.0 % of total protein) have been obtained (Robert Birch, T. Erik Mirkov, personal communications); however, in some cases it has been necessary to screen hundreds of independent lines to recover one superior line that produces these high levels. Our data indicate that transgenes introduced to sugarcane by particle bombardment frequently undergo a major decrease in expression after plant regeneration. In many cases, transgene expression remains detectable but at a level far lower than the same line produced at the callus stage. We observed this expression decrease similarly with sugarcane ubi4, ubi9, or maize *Ubi-1* promoters.

What accounts for the difference in frequency of PTGS between our rice and sugarcane lines? The rice and sugarcane lines differ in three major respects: species, whether the promoters are endogenous or foreign to the species, and method of transgene introduction. We are not aware of differences in PTGS between rice and sugarcane, but it is certainly possible that diploid rice may have evolved different mechanisms for responding to homologous DNA sequences than has highly polyploid sugarcane. As to the endogenous/foreign character of the promoters, other sugarcane promoters that became silenced when re-introduced into sugarcane have been reported (Birch et al. 1995, Hansom et al. 1999), however, there are also reports of endogenous sugarcane promoters that did not become silenced when re-introduced into sugarcane (Birch et al. 1995, Hansom et al. 1999). Additionally, the rice Act1 and cytochrome c (OsCc1) promoters have been used in transgenic rice without incurring silencing

(Zhang et al. 1991, Jang et al. 2002). Together these indicate that while homology to endogenous sequences may in some cases effect a transgene promoter, such homology does not preclude stable, unsilenced expression.

The method of transgene introduction, *Agrobacterium* co-cultivation vs. particle bombardment, certainly could influence the frequency of silencing. *Agrobacterium* transformation typically results in low- or single-copy transgene insertions, whereas particle bombardment typically produces multi-copy transgene arrays (Kohli et al. 1998, Butterfield et al. 2002). Transgene copy number *per se* has been shown to effect silencing in *Drosophila* (Sabl and Henikoff 1996), and copy number has been found related to silencing in plants (Hobbs et al. 1990, Matzke et al. 1994); however, this has not been demonstrated in sugarcane, where Hansom et al. (1999) found no correlation between transgene copy number and frequency of silencing in bombardment produced sugarcane. In sugarcane bombardment engineered for PTGS based virus resistance, resistant lines tended to have four to ten transgene copies, while susceptible lines tended to have either more or fewer copies (Ingelbrecht et al. 1999). In rice, Kohli et al. (1999) found no direct correlation between transgene copy number and silencing, but rather that aberrant transcripts, possibly derived from truncated transgenes, did correlate to silencing. While the effect of copy number on silencing remains unclear, the rearrangements frequently found in the multi-copy arrays resulting from bombardment (Kohli et al. 1998) certainly increase the likelihood of aberrant transcript production. Our nuclear run-off experiments indicated that pUC plasmid and *Ubi-1* promoter sequences were being transcribed in our sugarcane lines, and so it is quite possible that other unintended transcription events occurred. If such events resulted in the production of anti-sense, self-complementary «hairpin», or other aberrant RNA, this would be likely to result in PTGS of the transcribed sequence (Chuang and Meyerowitz 2000).

Current research on the mechanisms of PTGS has not reported a direct role for the promoter (for recent review see Voinnet 2001). This does not, however, rule out the possibility, and the work of Hansom et al. (1999) supports such a role. Our results, however, revealed no difference between the sugarcane *ubi* promoters and maize *Ubi-1* with regard to the frequency of silencing in sugarcane. Our results demonstrate that the sugarcane *ubi9* promoter compares favorably to maize *Ubi-1* for high-level constitutive expression in rice. Further research will be required to determine the possible utility of *ubi4* and *ubi9* in sugarcane.

References

- Albert HH, Carr JB, Moore PM (1995) Nucleotide Sequence of a sugarcane polyubiquitin cDNA (Accession #L41658). (PGR95–045). Plant Physiol 109: 337
- Bennett M, Smith J (1976) Nuclear DNA amounts in angiosperms. Phil Trans R Soc Lond 274: 227–274
- Birch RG, Bower R, Elliott A, Potier B, Franks T, Cordeiro G (1995) Expression of foreign genes in sugarcane. In: Cock JH, Brekelbaum T (eds) «International Society of Sugar Cane Technologists Congress XXII» Cartagena, Columbia. p368–373
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
- Butterfield MK, Irvine JE, Garza MV, Mirkov TE (2002) Inheritance and segregation of virus and herbicide resistance transgenes in sugarcane. Theor Appl Genet 104: 797–803
- Chamberlain DA, Brettell RIS, Last DI, Wirtzens B, McElroy D, Dolferus R, Dennis ES (1994) The use of the Emu promoter with antibiotic and herbicide resistance genes for the selection of transgenic wheat callus and rice plants. Austr J Plant Physiol 21: 95–112
- Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5: 213–218
- Christensen AH, Sharrock RA, Quail PH (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18: 675–689
- Chuang CF, Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. Proc Nat Acad Sci USA 97: 4985–4990
- Cornejo MJ, Luth D, Blankenship KM, Anderson OD, Blechl AE (1993) Activity of a maize ubiquitin promoter in transgenic rice. Plant Mol Biol 23: 567–581
- Gallo-Meagher M, Irvine JE (1993) Effects of tissue type and promoter strength on transient GUS expression in sugarcane following particle bombardment. Plant Cell Rep 12: 666–670
- Gallo-Meagher M, Irvine JE (1996) Herbicide resistant transgenic sugarcane plants containing the *bar* gene. Crop Sci 36: 1367–1374
- Hansom S, Bower R, Zhang L, Potier B, Elliot A, Basnayake S, Cordeiro G, Hogarth DM, Cox M, Berding N, Birch RG (1999) Regulation of transgene expression in sugarcane. In: Singh S, Kumar V (eds) «International Society of Sugar Cane Technologists Congress XXIII» New Delhi, India. p278–290
- Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6: 271–282
- Hobbs SL, Kpodar P, DeLong CM (1990) The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Mol Biol 15: 851–864
- Ingelbrecht I, de Carvalho F (1992) Isolation of nuclei and *in vitro* run-off transcription. In: Inze D, Van Der Straeten M, Van Montagu M (eds) Practical Course on Plant Molecular Biology. EMBO, Gent pp 117–132
- Ingelbrecht IL, Irvine JE, Mirkov TE (1999) Posttranscriptional gene silencing in transgenic sugarcane. Dissection of homology-dependent virus resistance in a monocot that has a complex polyploid genome. Plant Physiol 119: 1187–1198
- Jang IC, Choi WB, Lee KH, Song SI, Nahm BH, Kim JK (2002) High-level and ubiquitous expression of the rice cytochrome c gene OsCc1 and its promoter activity in transgenic plants provides a useful promoter for transgenesis of monocots. Plant Physiol 129: 1473–1481

- Jefferson RA, Burgess SM, Hirsch D (1986) β -glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc Nat Acad Sci USA* 83: 8447–8451
- Kohli A, Leech M, Vain P, Laurie DA, Christou P (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. *Proc Nat Acad Sci USA* 95: 7203–7208
- Kohli A, Gahakwa D, Vain P, Laurie DA, Christou P (1999) Transgene expression in rice engineered through particle bombardment: Molecular factors controlling stable expression and transgene silencing. *Planta* 208: 88–97
- Last D, Brettell R, Chamberlain D, Chaudhury A, Larkin P, Marsh E, Peacock W, Dennis E (1991) pEmu: an improved promoter for gene expression in cereal cells. *Theo Appl Genet* 81: 581–588
- Ma H, Albert H, Paull R, Moore P (2000) Metabolic engineering of invertase activities in different subcellular compartments affects sucrose accumulation in sugarcane cells. *Aust J Plant Physiol* 27: 1021–1030
- Matzke AJ, Neuhuber F, Park YD, Ambros PF, Matzke MA (1994) Homology-dependent gene silencing in transgenic plants: epistatic silencing loci contain multiple copies of methylated transgenes. *Mol Gen Genet* 244: 219–229
- McElroy D, Zhang W, Cao J, Wu R (1990) Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* 2: 163–171
- McElroy D, Blowers AD, Jenes B, Wu R (1991) Construction of expression vectors based on the rice actin 1 (Act1) 5' region for use in monocot transformation. *Mol Gen Genet* 231: 150–160
- Pelham HR (1982) A regulatory upstream promoter element in the *Drosophila* hsp 70 heat-shock gene. *Cell* 30: 517–528
- Sabl JF, Henikoff S (1996) Copy number and orientation determine the susceptibility of a gene to silencing by nearby heterochromatin in *Drosophila*. *Genetics* 142: 447–458
- Scharf K-D, Materna T, Treuter E, Nover L (1994) Heat stress promoters and transcription factors. In: Nover L (ed) *Plant Promoters and Transcription Factors*. Springer-Verlag, Berlin pp 125–162
- Schenk PM, Sagi L, Remans T, Dietzgen RG, Bernard MJ, Graham MW, Manners JM (1999) A promoter from sugarcane bacilliform badnavirus drives transgene expression in banana and other monocot and dicot plants. *Plant Mol Biol* 39: 1221–1230
- Schenk PM, Remans T, Sagi L, Elliott AR, Dietzgen RG, Swennen R, Ebert PR, Grof CP, Manners JM (2001) Promoters for pregenomic RNA of banana streak badnavirus are active for transgene expression in monocot and dicot plants. *Plant Mol Biol* 47: 399–412
- Singh GB, Kramer JA, Krawetz SA (1997) Mathematical model to predict regions of chromatin attachment to the nuclear matrix. *Nucleic Acids Res* 25: 1419–1425
- Takimoto I, Christensen AH, Quail PH, Uchimiya H, Toki S (1994) Non-systemic expression of a stress-responsive maize polyubiquitin gene (*Ubi-1*) in transgenic rice plants. *Plant Mol Biol* 26: 1007–1012
- Voinnet O (2001) RNA silencing as a plant immune system against viruses. *Trends Genet* 17: 449–459
- Wang J, Jiang J, Oard J (2000) Structure, expression and promoter activity of two polyubiquitin genes from rice (*Oryza sativa* L.). *Plant Sci* 156: 201–211
- Wei H (2001) Isolation and characterization of two sugarcane polyubiquitin gene promoters and matrix attachment regions, University of Hawaii, Manoa
- Wei H, Albert HH, Moore PH (1999) Differential expression of sugarcane polyubiquitin genes and isolation of promoters from two highly-expressed members of the gene family. *J Plant Physiol* 155: 513–519
- Wilmink A, van de Ven BC, Dons JJ (1995) Activity of constitutive promoters in various species from the Liliaceae. *Plant Mol Biol* 28: 949–955
- Zhang W, McElroy D, Wu R (1991) Analysis of rice Act1 5' region activity in transgenic rice plants. *Plant Cell* 3: 1155–1165